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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 5 :		(11) International Publication Number:	WO 93/13797
A61K 39/106, 47/48	A2	(43) International Publication Date:	22 July 1993 (22.07.93)

(21) International Application Number: PCT/US93/00253

(22) International Filing Date: 14 January 1993 (14.01.93)

(30) Priority data:
07/821,453 16 January 1992 (16.01.92) US

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(81) Designated States: AU, CA, JP, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).

Published
Without international search report and to be republished upon receipt of that report.

(54) Title: DETOXIFIED LPS-CHOLERA TOXIN CONJUGATE VACCINE FOR PREVENTION OF CHOLERA

(57) Abstract

A vaccine formulation comprising conjugates of detoxified LPS with proteins including cholera toxin (CT) is disclosed. Treatment with hydrazine (DeA-LPS) reduces the endotoxic properties of the LPS to clinically acceptable levels and results in a larger and more antigenic molecule than the saccharide produced by acid hydrolysis. The conjugates utilizing the cholera toxin of *V. cholerae* are disclosed which have low levels of pyrogen, no toxic activity upon Chinese hamster overay cells and elicit booster responses of vibriocidal and CT antibodies when injected subcutaneously as saline solutions into mice. The conjugates produced as a cholera vaccine induce the same antibodies as parenterally injected cellular vaccines but have improved safety and immunologic properties.

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Detoxified LPS-Cholera Toxin Conjugate Vaccine for Prevention of Cholera

FIELD OF THE INVENTION

The invention disclosed herein relates broadly to the production of vaccines for the amelioration of bacterial infections. More specifically, the invention describes the production of an antibacterial vaccine by conjugation of detoxified lipopolysaccharide derived from the bacterial target strain to proteins also produced by the bacteria target.

BACKGROUND ART

Cholera persists as a cause of illness and death in at least 40 countries on 3 three continents: ~340,000 cases have been reported in the Western hemisphere since an epidemic started in Peru, January 1991 [16,33]. Worldwide prevention of cholera by immunization has not been achieved because of the limitations of available vaccines. Research into new vaccines is difficult because there is no consensus as to the moieties which best elicit a protective immune response. The absence of bacterial invasion, the systemic symptoms and the lack of intestinal inflammation characterizing the disease have led to the understanding that cholera is a toxin-mediated disease of the luminal surface of the jejeunum, and to the notion that a local intestinal response is required for protective immunity [4,10-12,21,22,24,26,32,36,44].

The lipopolysaccharide (LPS) of *Vibrio cholerae* is considered to be a protective antigen [3,18,22,36,38,43,51,60] but the structures, pathogenic role and host moieties involved in protective immunity to cholera are incompletely understood. *V. cholerae* 01 LPS contains lipid A and a core oligosaccharide composed of 4-amino-4-deoxy-L-arabinose, quinovosamine, D-glucose, D-fructose and heptose [23,30,47]. 3-deoxy-D-manno octulosonic acid (KDO) has been identified recently and presumed to be in the core adjacent to the lipid A [5].

The O-specific polysaccharide (O-SP) of *V. cholerae* O, serotype Inaba, contains a saccharide of ~12 residues composed of 1→2-linked D-perosamine whose amino groups are acylated by 3-deoxy-L-glycero-tetronic acid [23,30,47]. The relationship between the sequence of the genes that encode the enzymes which synthesize the *V. cholerae* LPS and the serological specificity of the serotypes (LPS types) Inaba and Ogawa [23,36] has not been clarified.

Parenterally administered cellular vaccines or partially purified LPS induce a statistically significant protection against cholera in adults (~60%) for ~6 months [3,7,18,22,38,45]. Cellular vaccines are less effective for infants and young children and ineffective for control of outbreaks of cholera [38,51]. The protective immune moiety induced by these vaccines is proposed to be serum LPS antibodies with vibriocidal activity [1,3,18,22,38,40]. The cellular vaccines do not elicit serum antitoxin [37] nor, by analogy with similar products, secretory antibodies [56]. Similar effects are also obtained with orally administered inactivated *V. cholerae* [7,10-12]. Addition of the B subunit of CT to the formulation of this vaccine does not recruit additional protection [12].

Although considered by many workers as a "marker" and not as a protective moiety, vibriocidal antibody levels are a reliable method for predicting resistance to cholera. Serum vibriocidal activity is correlated with resistance against disease following convalescence from cholera, by administration of live attenuated strains, or by inactivated V. cholerae alone or with the B subunit of CT. Also, the age-related acquisition of vibriocidal antibodies in endemic areas parallels the increasing resistance to cholera observed in older children and adults [1,3,4,7,10,11,18,22,38,50]. Our interpretation of these data is that cellular cholera vaccines, as

observed with similar products and polysaccharides, are poor immunogens and have T-cell independent properties [37,49].

Based on similar reasoning, Kabir synthesized a bivalent conjugate composed of NaOH-treated LPS from serotypes Inaba and Ogawa bound to a protein extract of *V. cholerae* 395 (Ogawa) [27]. One mg of this conjugate, in complete Freund's adjuvant, elicited antibodies in rabbits with vibriocidal activity against the two serotypes. The route of immunization, using CFA, and the dosage used are clinically unacceptable.

Although the use of another component from *V. cholerae* may obscure the nature of protection elicited by our conjugates, we chose CT because it served as an immunogenic carrier for both the *H. influenzae* type b and the Vi polysaccharides [49,54]. Further, there remains the possibility that serum antitoxin, specific for the CT of the infecting strain, may be protective or exert synergistic protective activity with LPS antibodies [28, 43, 52].

Conjugate vaccines have many advantages compared to cellular vaccines; 1) no serious adverse reactions are anticipated because the LPS levels are low; 2) conjugated saccharides can be expected to have greater immunogenicity and T-cell dependent properties compared to cellular vaccines [9,14,31,48], thus the conjugate may represent a safer and more immunogenic (and thereby more effective) vaccine; 3) conjugates may be administered concurrently with Diptheria and Tetanus toxoid, Pertussis (DTP) and *H. influenzae* type b conjugates to infants [48], thus the conjugate might be incorporated into routine immunization of infants and children, that age group with the highest attack rate in areas endemic for cholera [38] and 4) the composition of our conjugate can be standardized so that the potency of new lots can be controlled by laboratory assays.

SUMMARY OF THE INVENTION

To solve the problems of adverse reactions, the lesser immunogenicity in infants and young children and the T-cell independent properties of the LPS in cellular vaccines, we synthesized conjugates, composed of detoxified LPS of *V. cholerae* serotype Inaba, to several proteins including CT [48]. The synthesis and immunologic properties of these vaccines, using CT as a carrier, are described infra.

The preparation of conjugates to elicit LPS antibodies is difficult because; 1) the complete structures of the LPS of the two serotypes are not known and; 2) the size of the Inaba O-SP is relatively small (approximately 6,000 daltons molecular weight (d)) (The immunogenicity of saccharides alone or in conjugates is directly related to their size) [2,17,54]. Treatment of LPS from strain 569B (Inaba) with 1% acetic acid, 100°C, for 90 minutes, results in a product of about 5,900 d, which has clinically acceptable levels of endotoxin but does not precipitate with hyperimmune sera. Treatment with hydrazine detoxifies the LPS to acceptable levels, resulting in products having molecular weights of approximately 13,000 and 6,000 d and retaining their antigenicity. Accordingly, the hydrazine-treated LPS is used to prepare the conjugates. As demonstrated for O-SP of *Shigella dysenteriae* type 1 [9], conjugates prepared by multipoint attachment (DeA-LPS-CTII) elicit higher levels of LPS antibodies than those prepared by single point attachment (DeA-LPS-CTI). Our conjugates, injected subcutaneously in saline at 1/10th the proposed human dose, elicit LPS antibodies with vibriocidal activity in young outbred mice. This immunization scheme was predictive of immunogenicity of *H. influenzae* type b-tetanus toxoid conjugates in infants injected concurrently with DTP [49]. The low levels of "endotoxic" activity, as measured by the LAL and rabbit

pyrogen assays, provide assurance that our conjugates will elicit little or no adverse reactions encountered with cellular vaccines for cholera [25].

The most general description of the invention is an anti-bacterial vaccine formulation which comprises a conjugate between lipopolysaccharide moieties derived from the target bacterial strain and proteins derived from the same strain. A vaccine is formulated using this conjugate and any of the pharmaceutically acceptable carriers, stabilizers, adjuvants and the like that are known in the art of vaccine preparation. Such a carrier may be sterile saline for the preparation of an injectable vaccine. The conjugates may also be incorporated into formulations currently in use in childhood immunization protocols, in particular, the diptheria and tetanus toxoid, pertussis (DTP) vaccine commonly administered to children in the United States.

A large advantage in terms of clinical usefulness of the conjugate vaccine is obtained by detoxifying the LPS component of the vaccine. Such detoxification can be achieved by removal of the esterified fatty acids from the lipid A component of the LPS using hydrazine or by acid hydrolysis of the LPS. Accordingly, one object of the present invention is to provide LPS-protein conjugate vaccines using such a detoxified LPS component.

Furthermore, it might be expected that conjugation of the detoxified LPS to a protein synthesized by the bacterial target would provide a useful vaccine, particularly if a protein localized on the surface of the bacterium is used. A vaccine which produces a neutralizing antibody response to toxins secreted by bacteria would also be a useful vaccine. Accordingly, it is a second object of the present invention to provide a vaccine wherein a detoxified LPS is conjugated to such a secreted toxin.

A preferred embodiment of the invention is the

conjugation of detoxified LPS to a toxin protein produced by the bacterial target.

The conjugation reaction can be carried out using a variety of reagents. The conjugation can be directly between the LPS and the protein or carried out using a cross-linking agent. Such a cross-linking agent can be a bifunctional linker. Examples of bifunctional linkers which can be employed in the present invention include, but are not limited to, adipic acid dihydrazide, diaminohexane, amino-ε-caproic acid, and an N-hydrosuccinimide acid anhydride-based heterobifunctional linker.

As preferred embodiments, two methods of conjugation are described for the LPS detoxified by treatment with hydrazine; reaction with N-succinimidyl 3-(2-pyridyldithio) propionate (SPDP) or reaction with adipic acid dihydrazide (ADH) followed by reaction with 1-ethyl-3(3-dimethylaminopropy) carbodiimide (EDAC). The latter method results in the formation of a covalently bonded aggregate (lattice) of the LPS-protein conjugate. A preferred embodiment of the invention utilizes one of these methods of conjugation.

The polyclonal or monoclonal antibodies raised by administration of the conjugate vaccine to a laboratory animal may find use as components of a diagnostic kit or as components of a method of treatment of infection by the targeted organism. Accordingly, it is another object of the invention to provide for diagnostic kits for the detection of organisms bearing either the LPS or protein portion, or both, of the conjugate. A final object of the invention is to provide antibodies which might be used to treat infections caused by an organism bearing either the LPS or protein portion, or both, of the conjugate or which neutralize a toxin secreted by such an organism.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows a silver-stained SDS-PAGE gel (14%) of 2.5 mg LPS from *Vibrio cholerae* serotype Inaba (lane 1) and Escherichia coli O111 (lane 2).

Figure 2 shows the characterization of LPS and LPS-CT conjugates by double immunodiffusion. Left: A. Hyperimmune *Vibrio cholerae* serotype Inaba serum, outer wells: 1 - Inaba LPS, 250 mlml, 2 - Inaba DeA-LPS, 250 mlml, 3 - Inaba O-SP, 250 mlml. Right: A. Hyperimmune *V. cholerae* serotype Inaba serum, B. hyperimmune cholera toxin antiserum, 3 - Inaba DeA-LPS, 250 mlml, 4 - Conjugate DeA-LPS-CTII.

Figure 3 shows ^{13}C nuclear magnetic resonance spectrum of the hydrazine-treated lipopolysaccharide (DeA-LPS) from *Vibrio cholerae*, serotype Inaba. The 10 major signals are identical to those reported by Kenne et al., [30]. The ^{13}C N.M.R. spectrum of the acid-treated lipopolysaccharide (O-SP) was almost identical to this spectrum.

Figure 4 shows HPLC profiles of 100 mL samples (1.0 mg/ml) through a 10x300 mm column of Superose 12 in 0.2 M NaCl, 0.01 M TRIS, 0.001 M EDTA, 0.25% deoxycholic acid, pH 8. a. LPS serotype Inaba; b. DeA-LPS Inaba; c. O-SP Inaba.

DISCLOSURE OF THE INVENTION

Scientific papers and other literature cited in this application are incorporated herein by reference in their entirety.

Preferred embodiments of the invention are described in detail below by means of representative examples. These examples are meant only as illustrations of the invention and are not to be taken as limiting of the scope of the invention.

Chemical reagents for executing the procedures described in the examples can be obtained from the sources noted below:

Anhydrous hydrazine (Lot 104F-3523), adipic acid dihyrazide (ADH, Lot 77F-5016), dithiothreitol (DTT, Lot 49F-0138), 1-ethyl-3(3-dimethylaminopropyl) carbodiimide (EDAC, Lot 105F-0308), disodium EDTA (Lot 119F-0275), KDO, RNase (Lot 128F-0462), DNase (Lot 89F-9605) and pronase (Lot 99F-0391) can be purchased from Sigma Chemical Co., St. Louis, MO. HEPES (Lot 051790) and deoxycholic acid (Lot 264101) can be purchased from Calbiochem, La Jolla, CA. N-succinimidyl 3-(2-pyridyldithio) propionate (SPDP, Lot 900707084), alum (Lot 891120103) and BCA reagent for protein determination can be obtained from Pierce Chemical Co, Rockford, IL. Cyanogen bromide (CNBr, Lot 014783A) can be purchased from Eastman Chemical, Rochester, NY. Sephadex G-25 (Lot P10036), Sephacryl S-300, 10x300 mm Superose 12 column and dextrans for molecular weight assay can be purchased from Pharmacia-LKB, Piscataway, NJ. LPS from *V. cholerae* strain 569B (Inaba) can be purchased from List Biologicals, Campbell, CA. Limulus amoebocyte lysate (LAL) can be purchased from Associates of Cape Cod, Woods Hole, MA. p-nitrophenyl phosphate can be obtained from Fluka, Ronkonkoma, NY. The US Standard for endotoxin can be obtained from Donald Hochstein, United States Food and Drug Administration [25]. Cholera toxin, variant 1, Lot 582 can be obtained from Pasteur Merieux Serums & Vaccins, Lyon, France) and cholera toxin variant 1, lot rst is purified from *V. cholerae* Inaba strain 569B [21,28]. Anti-mouse IgG and IgM alkaline phosphatase conjugates can be purchased from Kirkegaard & Perry Laboratories, Inc, Gaithersburg, MD.

The bacterial strains used in the examples are: *V. cholerae*, biotype classical, serotype Inaba, strain 569B and *V. cholerae*, biotype classical, serotype Ogawa strain NIH 41 are used for vibriocidal assay. *V. cholerae*, classical Inaba strain 2524 (Katherine Greene, C.D.C., Atlanta, GA) is used for raising antiserum in mice

against the LPS. *V. cholerae*, serotype Inaba, biotype El Tor, cholera toxin (CT) variant 2, strain 075, is a recent isolate from South America (Richard Haberberger, Naval Research Medical Institute, Bethesda, MD). All of these strains may be obtained by contacting the laboratory of Dr. Shousun Szu, Laboratory of Developmental and Molecular Immunity, National Institute of Child Health and Human Development, National Institutes of Health, Bethesda, MD 20892 or the laboratory of Dr. John B. Robbins at the same address. Equivalent strains may be obtained from the American Type Culture Collection, Rockville, MD. These strains are catalogued as *Vibrio cholerae* (Inaba), ATCC 9459 and *Vibrio cholerae* (Inaba, biotype El Tor) ATCC 14033.

Example 1: Production and characterization of detoxified lipopolysaccharides

LPS is detoxified by two methods. For acid-hydrolysis, LPS, 10 mg/ml in 1% acetic acid is heated at 100°C for 90 min [59]. The reaction mixture is ultracentrifuged at 60,000 x g, 10°C, for 5 hr. and the supernatant passed through a sterile 0.22 micron filter (Nalge, Rochester, NY) and freeze-dried (designated as O-SP). For detoxification by base-hydrolysis, LPS at 10 mg/ml, is treated with hydrazine at 37°C for 2 hr. Hydrazine treatment has been reported to remove esterified fatty acids from the lipid A, accordingly this product is designated as DeA-LPS. This material is mixed with acetone in an ice bath until a precipitate formed (approximately 90% acetone) and the reaction mixture centrifuged at 15,000 x g, 10°C, for 30 min. The precipitate is dissolved in 0.15 M NaCl, pH 7.0 to about 3 mg/ml. The reaction mixture is centrifuged at 60,000 x g for 5 hr. at 10°C, the supernatant dialyzed against H_2O exhaustively, passed through a 0.22 micron filter, and

freeze dried. The protein and nucleic acid concentration of the O-SP and the DeA-LPS are <1%. LPS, extracted from acetone-dried *V. cholerae* cells of El Tor biotype Ogawa serotype strain 3083-13, is used for inhibition of vibriocidal activity.

The LPS is subjected to various preliminary characterizations, using both in vitro and in vivo techniques. SDS-PAGE is used for detection of LPS [56]. LPS concentration, assayed by LAL, is expressed in endotoxin units (EU) related to the US standard [25]. The molecular sizes of LPS, O-SP and deacylated LPS (DeA-LPS) are estimated by gel filtration through Superose 12 in 0.2 M NaCl, 1 mM EDTA, 10 mM Tris, 0.25% deoxycholic acid, pH 8.0, using the dextran standards to calibrate the column. KDO is measured by the thiobarbituric acid assay using KDO as a standard [5]. Double immunodiffusion is performed in 1% agarose in phosphate-buffered saline (PBS). Data for NMR spectra are recorded on a JEOL GSX-500 spectrometer. Each spectrum is acquired with broad-band ^{1}H decoupling at 90° 10-msec carbon observed pulse; 32,000 data points which are zero-filled to 64,000 points prior to Fourier transformation; 30 KHz spectral window (0.54 sec acquisition time); 3.0 sec delay between pulse cycles. Prior to Fourier transformation each free-induction-decay signal is exponentially multiplied so as to result in an additional 4Hz line-broadening in the frequency domain spectrum. Approximately 10 mg of each sample are dissolved in 0.5 mL of D_2O and recorded at ambient probe temperature (21°C).

Silver-stained SDS PAGE of 2.5 mg of LPS from Inaba shows 2 faint bands with a "ladder" in the middle and two dense bands near the bottom of the gel (Fig. 1). Typical "ladders" of higher molecular weight O-SP are formed by the LPS from E. coli 0111. No bands are observed with 10 mg samples of either the O-SP or the DeA-LPS. The LPS of

serotype Inaba has 3-6x10^3 EU/mg and the DeA-LPS had 3 EU/mg by LAL assay: this level represents >1000-fold reduction. Immunodiffusion shows a single band of precipitation between the LPS and the hyperimmune LPS sera (Fig. 2). A less intense and more diffuse band is observed with the DeA-LPS which yields a partial identity reaction with the LPS. Neither the O-SP nor the CT precipitates with this hyperimmune serum. The molecular sizes of the LPS, O-SP and DeA-LPS are estimated by HPLC on Superose 12 (Fig. 3). The LPS and DeA-LPS show two peaks: the LPS has Kd values of 0.40 (16,000 d) and 0.46 (8,700 d) and the DeA-LPS has Kd values of 0.38 (13,000 d) and 0.50 (6,000 d). The O-SP exhibited only one peak corresponding to the second peak of the DeA-LPS (Kd 0.51, 5,900 d). Because of its greater antigenicity and high molecular weight, DeA-LPS is preferably used as the saccharide for the conjugates. We cannot detect KDO in either the O-SP or the DeA-LPS by the thiobarbituric acid assay [5,57]. The ^{13}C NMR spectra of the DeA-LPS and O-SP are in agreement with previous reports [30,47]. Each spectrum shows 10 major signals with identical, or nearly identical, chemical shifts to those reported (Fig. 3).

Example 2: Production and characterization of DeA-LPS-Cholera Toxin conjugates

Conjugation of DeA-LPS with proteins is performed using either of two methods. In method 1, the covalent attachment of the LPS to the protein is accomplished by using SPDP to thiolate both the protein and the DeA-LPS as described for the cell wall polysaccharide of pneumococci [52]. DeA-LPS (3 mg/ml) or protein (10 mg/ml) are dissolved in 0.15 M HEPES, 2 mM EDTA, pH 7.5. SPDP (20 mM in ethanol) is added dropwise at weight ratios of 0.5 for DeA-LPS and 0.2 for protein. The

reaction mixture is stirred at ambient temperature for 1 hr. and passed through a 5x35 cm Sephadex G-25 column in H_2O for DeA-LPS-SPDP and in PBS for protein. The DeA-LPS-SPDP is freeze-dried and the protein is concentrated by membrane filtration (Amicon, YM10). The extent of derivatization with SPDP in aliquots of DeA-LPS or the proteins is determined spectrophotometrically following reduction of the N-pyridyl disulfide bond with 40 mM DTT and assuming a molar extinction coefficient at 340 nm of $8.08x10^4$ [53]. The N-pyridyl disulfide on the DeA-LPS-SPDP is reduced with 40 mM DTT, passed through a 2.5x50 cm column of G-25 Sephadex in 0.2 M NaCl and the void volume fractions mixed with the SPDP derivative of the protein. This reaction mixture is stirred at room temperature for 2 hrs., passed through a 5x100 cm column of S-300 Sephacryl in 0.2 M NaCl and the void volume fractions pooled. The conjugate synthesized by this method using Cholera Toxin as the protein component is designated as DeA-LPS-CTI. An aliquot of DeA-LPS-CTI in saline is treated with 0.05 M EDAC at room temperature for 1 hr. at pH 6.0 to cross-link the conjugate. The non-reacted EDAC is removed by exhaustive dialysis against water.

In method 2, DeA-LPS is derivatized with ADH as described for *Haemophilus influenzae* type b [9,48]. DeA-LPS, 10 mg/ml in saline, is brought to pH 10.5 with 1 N NaOH and an equal weight of CNBr (1 g/ml in acetonitrile) is added. The pH is maintained between 10.0 and 11.0 with 1 N NaOH for 3 minutes. An equal volume of 0.5 M ADH in 0.5 M $NaHCO_3$ is added and the pH adjusted to 8.5. The reaction mixture is stirred at room temperature for 1 hr. and then at 3-8°C overnight and passed through a 5x35 cm Sephadex G-25 column in H_2O. Fractions from the void volume are pooled and freeze dried. The DeA-LPS-AH derivative is dissolved in 0.15 M NaCl to 10 mg/ml. An equal volume of protein (~10 mg/ml) is added and the pH

adjusted to 5.5 with 0.1 M HCl. EDAC is added to a final concentration of 0.05 M and the pH is maintained at 5.5-6.0 for 1 hr.. The reaction mixture is passed through 2.5x90 cm column of S-300 Sephacryl in 0.2 M NaCl and the fractions in the void volume are pooled. Conjugates synthesized using CT (Lot 582) and CT (Lot rst) as the protein component are designated as DeA-LPS-CTII and DeA-LPS-CTIII.

As in example 1, the conjugates are characterized by several in vitro and in vivo methods. The extent of derivatization of DeA-LPS with adipic acid hydrazide is measured by reaction with trinitrobenzene sulfonic acid (TNBS) using ADH as a standard [9]. Protein is measured by the BCA reagent using bovine serum albumin as a standard [17]. Hexose is measured by the anthrone reaction using the O-SP as the standard [55]. Endotoxin concentration is estimated as in Example 1. Double immunodiffusion is also performed as in Example 1. In vitro cytotoxicity of CT is measured by observation of elongation of CHO cells [15]. Pyrogenicity of the conjugate is assayed in rabbits using the method of Hochstein et al. [25].

Table 1 shows some of the results of characterization of the conjugates.

Table 1. Characterization of *Vibrio cholerae* hydrazine-treated lipopolysaccharide-protein conjugates.

Conjugate	Linker	DeA-LPS (wt/wt)	protein (wt/wt)	Yield %	DeA-LPS/ protein wt/wt	Composition mg/ml	
						protein	CHO_
DeA-LPS-CT_I	SPDP	1.18	8.8	88	0.72	2.0	1.44
DeA-LPS-CT_I*	SPDP	1.18	8.8	NA	0.65	1.15	0.25
DeA-LPS-CT_{II}	ADH	1.76	NA	79	0.80	0.48	0.38
DeA-LPS-CT_{III}	ADH	1.76	NA	88	1.50	1.0	1.50

*Further treated with 0.05M with EDAC (MATERIALS AND METHODS)

NA: not applicable

Polysaccharide measured by anthrone reaction with DeA-LPS as a standard [ ]. The yield was calculated based upon the weight of the saccharide in the conjugate compared to the starting weight of the adipic acid hydrazide derivative.

The extent of derivatization of the DeA-LPS with SPDP or with ADH, is similar. The DeA-LPS/protein (wt/wt) ratios are slightly lower for the conjugates of CT prepared with SPDP than with ADH, ranging from 0.72 for DeA-LPS-CTI to 1.5 for DeA-LPS-CTII. The yields for all the conjugates are ~80% as calculated by the recovery of saccharide in the conjugates compared to the derivatized polysaccharide. Similar results are obtained by Method II with tetanus toxoid as the protein component of the conjugate. A representative double immunodiffusion experiment shows that the serotype Inaba hyperimmune antiserum yields an identical line of precipitation with the DeA-LPS and DeA-LPS-CTII (Fig. 2). Similarly the CT and LPS antisera yields a line of identity with the DeA-LPS-CTII and the CT. A faint spur from the CT antiserum extends over the LPS antiserum and the conjugate, suggesting that there is a slight amount of unbound CT in this preparation. The residual toxicity of the CT and the DeA-LPS in the conjugates as estimated by the in vitro and in vivo assays described above is very low. In the thermal induction test, the DeA-LPS was not pyrogenic when injected at 1 mg/Kg rabbit body weight. The endotoxin content of the conjugates was ~2 EU/mg by the LAL assay. CT induced elongation in CHO cells at 0.4 ng/ml. The amount of CT, as a conjugated form required to elicit the same degree of elongation was 10^3 to 10^{10} greater. DeA-LPS-CTIII, a preparation intended for clinical use, had no detectable toxicity in CHO cell assay at 1.0 mg/ml and passed the general safety test in guinea pigs at 10 human doses (25 mg DeA-LPS per dose) as described in the Code of Federal Regulations C.

Example 3: Comparison of efficacy of cellular and LPS-CT conjugate vaccines

Hyperimmune LPS antiserum is prepared by injecting

female, adult BALB/c mice with heat-killed *V. cholerae* strain 2524 [41]. Burro CT antiserum is prepared as described [13]. For evaluation of immunogenicity, 6-weeks-old BALB/c or general purpose mice (NIH) are injected subcutaneously with 2.5 mg or 10 mg of DeA-LPS alone or as a conjugate in saline. Mice are injected at 2 week intervals and bled 7 days after each immunization. The fourth dose is given 4 weeks after the third injection and mice are bled 7 days and 6 months later. Groups of mice are immunized similarly with conjugates adsorbed with 0.125 or 1.25 mg of aluminum hydroxide per dose. Cellular cholera vaccine (purchasable from Wyeth-Ayerst Laboratories, Marietta, PA) containing $4x10^9$ each of Inaba and Ogawa serotypes, is used as a control. Mice are immunized with 0.1 or 0.2 ml of the vaccine.

Complement-mediated vibriocidal antibody is measured against Inaba and Ogawa strains [19,20]. Ten-fold serum dilutions are mixed with equal volumes of ~1000 cells/ml diluted guinea pig serum and incubated at 37°C for 1 hr. A hyperimmune serum is used as standard in each assay. The serum titer is expressed as the reciprocal of the highest dilution of serum that yielded 50% vibriocidal activity. Some sera were assayed for vibriocidal antibodies against strains 569B and 075 of serotype Inaba: the titers of these sera were identical against both strains. Therefore, vibriocidal activities of the sera are assayed with strain 569B. Inhibition of vibriocidal activity is assayed by mixing 100 mg/ml of LPS, DeA-LPS, O-SP or CT with various dilutions of antisera at 37°C for 1 hr. prior to the addition of the bacteria [20].

LPS and protein antibody levels are determined by enzyme-linked immunosorbent assay (ELISA) using Immunolon 4 plates (Dynatech, Chantilly, VA). The plates are coated with 100 ml per well of either LPS, 10 mg/ml, or CT, 5 mg/ml, in phosphate buffered saline (PBS). LPS

antibody levels are expressed in ELISA units using hyperimmune sera as a reference. CT antibody levels are expressed in ELISA units with a hyperimmune mouse pooled standard sera prepared using methods typically known in the art by repeated immunization of mice with CT. Antibody levels are expressed as the geometric mean. Antibody concentrations below the sensitivity of the ELISA are assigned values of one-half of that level. Comparison of geometric means is performed with the two-sided *t* test and the Wilcoxon test.

There are no detectable LPS antibodies in either general purpose or BALB/c mice immunized with 2.5 or 10 mg of DeA-LPS alone after any injection. Table 2 shows the levels of antibodies to LPS in general purpose mice immunized with DeA-LPS-CTI and DeA-LPS-CTII.

Table 2. Serum IgM and IgG LPS antibodies (ELISA) elicited in general purpose mice immunized with DeA-LPS alone or as conjugates[a]

Vaccine	Dose (μg)	Inject. Number	n=	Geometric mean (25-75th centiles) IgG	IgM
LPS	2.5	2	10	<10	320
		3	9	320	640
LPS	10.0	2	3	<10	50 (10-160)
		3	10	149[c] (5-2153)	260 (40-1522)
		4	10	1085[d] (80-12800)	1525 (269-5120)
DeA-LPS-CT_I	2.5	2	5	<10	<10
		3	8	22[e] (5-95)	11 (5-24)
		4	5	318[f] (7-20239)	152 (10-2263)
DeA-LPS-CT_{II}	2.5	2	5	35 (14-80)	23 (14-40)
		3	11	80[g] (20-320)	150 (20-640)
		4	1	1540[h] (320-12800)	219 (40-640)
		4[b]	5	640[i] (453-905)	80 (40-226)

[a]Female general purpose mice, ~6 wks-old, were injected s.c. with saline solutions of the antigen every week for three times and then were given a fourth injection 4 weeks later.

[b]The mice were bled 7 days after each injection and then again 6 months after the fourth injection.

h *vs* f, h *vs* d, p=NS; h *vs* g, p=0.002, d *vs* c, p=0.08; f *vs* e, p=0.06, h *vs* i, NS

Neither conjugate elicits LPS antibodies after the first immunization. DeA-LPS-CTII elicits IgG and IgM antibodies after the second injection. Both conjugates elicit a significant rise of IgG antibodies after the third and fourth injections ($P<0.01$). The IgG levels after fourth injection are similar in mice injected with either the LPS or DeA-LPS-CTII. LPS doses of 2.5 or 10.0 mg elicit IgG antibodies only after the third injection. The IgG levels are similar in the sera taken 7 days or 6 months after the fourth injection of DeA-LPS-CTII. Similar levels of antibodies are elicited by 10 mg doses of the conjugates, by EDAC treated DeA-LPS-CTI and by conjugates adsorbed onto alum.

Table 3 shows that the LPS antibody levels elicited by conjugates in BALB/c mice are lower than those of the general purpose mice.

Table 3. Serum IgM and IgG LPS anti-LPS antibodies elicited in BALB/c mice immunized with DeA-LPS-CT conjugates or cellular cholera vaccine

Vaccine	Dose (μg)	Inject. Number	n=	Geometric mean (25-75th centiles) IgG	IgM
DeA-LPS-CT_I	2.5 μg	1	5	<10	40 (40-40)
		2	5	<10	40 (40-40)
		3	10	<10	53 (40-40)
		3*	10	13 (5-24)[a]	65 (40-160)
DeA-LPS-CT_{II}	2.5 μg	1	5	<10	30 (20-40)
		2	5	<10	70 (40-160)
		3	10	46 (5-761)	130 (40-640)
		3*	6	32 (10-95)[b]	50 (40-80)
Whole-cell	0.1 ml	1	5	<10	106 (57-226)
		2	5	139 (40-640)[c]	1114 (453-2560)
		3	9	1742 (905-2560)[d]	741 (400-1600)
		3*	6	90 (67-160)[e]	101 (67-190)

* mice bled 5 months after the third immunization

e *vs* a, P=0.0004, e *vs* b, P=NS, e *vs* d, P=0.0001, d *vs* c, P=0.02

After the first and second dose, there is low IgM and no detectable IgG antibodies in BALB/c mice injected with the conjugates. Low levels are detected after the third injection. The antibody levels remain similar 5 months after the last injection. The cellular vaccine induces high levels of both IgG and IgM after the second injection and a booster effect upon the IgG antibody levels following the third injection. Dosages of 0.1 ml and 0.2 ml elicit similar levels. The IgG levels of the mice injected with the cellular vaccine decline to ~1/20 of their optimal values 5 months after the last injection ($P=0.0001$) and are similar to those elicited by DeA-LPS-CTII.

Neither 2.5 mg nor 10 mg of DeA-LPS or PBS (controls) elicits vibriocidal antibodies to *V. cholerae* serotypes Inaba or Ogawa. As shown in Table 4, DeA-LPS-CTII elicits low levels of vibriocidal antibodies to the Inaba strain in general purpose mice after the first injection.

Table 4. Vibriocidal antibody titers of pooled sera from NIH general purpose mice immunized with conjugates or LPS

Immunogen	Dose (μg)	Challenge serotype	Immunization number 1	2	3
LPS	10.0	Inaba	ND	50,000	500,000
DeA-LPS-CT_I	2.5	Inaba	ND	500	50,000
	2.5	Ogawa	ND	100	50,000
DeA-LPS-CT_{II}	2.5	Inaba	100	25,000	50,000
	2.5	Ogawa	<10	1,000	25,000
	10.0	Inaba	500	25,000	100,000
	10.0	Ogawa	50	1,000	50,000

* ND - Not done

General purpose mice from the NIH were injected s.c. with 2.5 μg of DeA-LPS and their sera were pooled in equal amount for each group

Both DeA-LPS-CTI and DeA-LPS-CTII elicit booster responses after the next two injections. LPS elicits the highest level of vibriocidal antibodies. In BALB/c mice, both conjugates elicit vibriocidal antibodies after the first injection; only DeA-LPS-CTII elicits booster responses following the second and third injections (Table 5).

Table 5. Vibriocidal activities of sera from BALB/c mice immunized with DeA-LPS alone, conjugated to cholera toxin (CT) or with whole cell cholera vaccine

Vaccine	Dose	Target	Reciprocal vibriocidal titer		
			1st inj	2nd inj	3rd inj
DeA-LPS-CT_I	2.5 μg	Inaba	100	100	5,000
		Ogawa	25	100	1,000
DeA-LPS-CT_{II}	2.5 μh	Inaba	250	5,000	100,000
		Ogawa	100	500	50,000
Whole cell	0.1 ml	Inaba	2,500	50,000	100,000
		Ogawa	25,000	500,000	1,000,000

+ Vibriocidal antibody titer of pooled sera after each dose

* Whole cell cholera vaccine

None of our conjugates elicits IgG antibodies following the first injection. This apparent lesser immunogenicity of DeA-LPS-CT conjugates, compared to those with capsular polysaccharides [48], could be due to two factors: 1) the lesser immunogenicity of the O-SP of *V. cholerae* 01 is due to its simplicity (linear homopolymer of perosamine acylated by 4-amino-4,6-dideoxy-L-glycero-tetronic acid) [30,47]; 2) the relatively low molecular weight of the O-SP of *V. cholerae* 01 [36,46].

We propose the following mechanism by which serum vibriocidal antibodies prevent cholera, a disease which is caused by a non-invasive organism, whose symptoms are mediated by an exotoxin and which is not accompanied by inflammation.

First, serum antibodies, especially those of the IgG class, penetrate into the lumen of the intestine [28,59]. It is likely complement proteins are also present. Second, the walls of the intestine are in contact due to peristalsis. Third, the inoculum that survives the acid conditions of the stomach is probably approximately 10^3 *V. cholerae* [22,31]. Fourth, *V. cholerae* have short polysaccharides on their LPS; this trait is associated with a high susceptibility to the complement-dependent action of serum antibodies [42]. These factors, namely low inocula, serum vibriocidal antibodies and complement at the mucosal surface whose surfaces are pressed upon each other and churning a susceptible organism, provide an explanation for how serum vibriocidal antibodies confer protection against cholera; ingested *V. cholerae* are lysed on the intestinal mucosal surface.

The conjugates elicit higher vibriocidal activity to the homologous serotype (Inaba) than to the heterologous serotype (Ogawa). The cellular vaccine, which contains both serotypes, induces higher levels of vibriocidal antibodies against Ogawa than Inaba. The vibriocidal

levels to serotype Inaba are elicited earlier and in higher titer by the cellular vaccine compared to the conjugates. After the third injection, the vibriocidal levels to Inaba elicited by the whole cell and conjugate vaccines are similar. All vibriocidal activity is removed from the conjugate-induced antibodies following adsorbtion with either the LPS, DeA-LPS or the O-SP of the Inaba serotype. Adsorption with the Inaba LPS also removes all of the vibriocidal activity from the sera of mice injected with the cellular vaccine. The DeA-LPS and O-SP, in contrast, removes approximately 90% of the vibriocidal activity from these sera. Absorption with the Ogawa LPS removes about 90% of the vibriocidal activity against strain Inaba. Absorption with CT does not change the vibriocidal titers from the sera of mice injected with either the conjugates or the cellular vaccines. Adsorption of DeA-LPS-CTI, DeA-LPS-CTII or other conjugates onto alum has no effect upon their immunogenicity.

Table 6 shows the cholera toxin antibodies that are induced by immunization with the conjugates. Significant rises of CT antibodies are elicited in all mice of both strains by both conjugates after each injection.

Table 6. Serum IgG cholera toxin antibodies in mice immunized with DeA-LPS conjugates (Geometric mean (25-75th centiles)

Vaccine	Injection number	Strain of mice	
		BALB/c	General purpose
DeA-LPS-CT_I	1	0.1[a](0.1-0.2)	0.1[f](0.1-0.2)
	2	56.9[b] (44-76)	49.0[g] (37-69)
	3	217.4[b]5;52-270)	157.1[h](134-198)
DeA-LPS-CT_{II}	1	0.1[c](0.1-0.2)	0.1[i](0.03-0.3)
	2	30.6[d] (11-80)	17.8[j] (11-31)
	3	136.7[e](102-198)	156.0[k](105-201)

* Mice immunized with whole cell cholera vaccine, DeA-LPS or PBS had <0.01 ELISA antibody levels.

b *vs* a, P=<0.001; d *vs* c, P=0.0001; e *vs* d, P=0.04; h,g *vs* f, P<0.01; b *vs* e, P=0.01; j,k *vs* i, P<0.01.

Example 4: Diagnostic and Therapeutic Applications

The LPS-protein conjugate of the present invention may be administered to human or animal subjects in the form of a vaccine for the purpose of treating or preventing infections caused by organisms containing the LPS and/or protein antigen. Such vaccines can contain approximately 5 to 100 μg of the LPS-protein conjugate. These vaccines can be administered subcutaneously or intramuscularly. The antibodies raised to the conjugate can be introduced into sterile filtered or radiation sterilized milk (bovine, ovine or caprine) and administered orally. The conjugates can be suspended in alum, saline, buffered saline, or oil-water emulsions, and subjects can be vaccinated with a series of injections, preferably one to five injections over a twelve month period.

Monoclonal or polyclonal antibodies of human or animal origin can be produced via the use of the above-described vaccines. These antibodies can be administered to animals and humans, alone or in combination with the LPS-protein conjugate vaccines of the present invention, for the prevention or treatment of infections caused by the organism(s) from which the LPS and protein components of the conjugate are obtained. These antibodies can be administered to a subject in need thereof, either alone, for the purpose of passive immunization, or in combination with the LPS-protein conjugate vaccines of the present invention, as an adjunct therapy. Such antibodies can take the form of serum or gamma globulin containing the antibodies of interest.

Of particular interest are antibodies to the LPS and CT antigens of *V. cholerae*. These antibodies demonstrate neutralizing activity against related bacterial toxin antigens, particularly the toxins secreted by *Eschericia coli*, *Campylobacter jejeuni* and *Aeromonas hydrophilia*.

Monoclonal or polyclonal antibodies prepared via the use of the conjugate vaccines of the present invention can also be used for diagnostic purposes, or for the investigation of the developmental processes, pathogenesis, prevention, immunopathology, of LPS alone, as a component of a complex molecule, such as the LPS-protein conjugate, or of organisms expressing this polysaccharide fragment or derivatives thereof. The antibodies can also be used to investigate the immunologic responses to the above antigens.

Such antibodies can be derivatized or reacted with other substances to produce kits for disease diagnosis, or for the identification of organisms containing the LPS or protein used in the conjugate.

The invention being thus described, it will be obvious that the same may be varied in many ways. Such variations are not to be regarded as a departure from the spirit and scope of the invention, and all such modifications as would be obvious to one skilled in the art are intended to be included within the scope of the following claims.

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WHAT IS CLAIMED IS:

1. A vaccine comprising purified, isolated bacterial lipopolysaccharide (LPS) which is detoxified and so exhibits low pyrogenicity in a mammal; and a pharmaceutically acceptable carrier.

2. The vaccine according to claim 1, in which the LPS has been detoxified by reaction with hydrazine.

3. The vaccine according to claim 1, in which the LPS has been detoxified by acid hydrolysis.

4. A conjugate compound comprising the LPS according to claim 1 covalently attached to a protein isolated from or secreted by a bacterial strain by means of a bifunctional linker.

5. A conjugate according to claim 4, wherein said protein is a cell surface localized protein.

6. A conjugate according to claim 4, wherein said protein is a bacterial toxin.

7. A conjugate according to claim 6, wherein said LPS and said bacterial toxin are obtained from the same organism.

8. A conjugate according to claim 6, wherein said organism is *Vibrio cholerae*.

9. A conjugate according to claim 7, wherein said toxin is the cholera toxin of *Vibrio cholerae* and said organism is *Vibrio cholerae*.

10. A conjugate according to claim 4, wherein said covalent attachment is accomplished by reaction with a bifunctional linker selected from the group consisting of adipic acid dihydrazide, diaminohexane, amino-ϵ-caproic acid, and an N-hydrosuccinimide acid anhydride-based heterobifunctional linker.

11. A conjugate according to claim 10, wherein said N-hydrosuccinimide acid anhydride-based heterobifunctional linker is N-succinimidyl-3-(2-pyridyldithio) propionate.

12. A covalent aggregate of LPS-protein conjugate comprising the conjugate of claim 10, wherein adipic acid dihydrazide reacted components are further reacted with 1-ethyl-3(3-dimethylaminopropyl) carbodiimide.

13. A method for preparing a covalent aggregate of LPS-protein conjugate comprising:

1) detoxifying the LPS component of the conjugate,

2) reacting the detoxified LPS component of step (1) with adipic acid dihydrazide,

3) mixing the derivatized, detoxified LPS from step (2) with the protein component of the conjugate,

4) adding to the mixture of step (3) 1-ethyl-3(3-dimethylaminopropyl) carbodiimide, and

5) collecting the covalent aggregate of LPS-protein conjugate.

14. The method of claim 13, wherein the LPS is detoxified in step (1) by reaction with hydrazine.

15. A vaccine according to claim 1, which further comprises a second vaccine.

16. A vaccine according to claim 15, wherein said second vaccine is a currently available DTP vaccine.

17. A vaccine comprising the conjugate of claim 4, and a second vaccine.

18. The vaccine of claim 17, wherein said second vaccine is a currently available DTP vaccine.

19. A method for immunizing a patient against cholera which comprises administering an amount of the vaccine of claim 1, sufficient to provide protection against cholera, to a patient.

20. A method for immunizing a patient against cholera which comprises administering an amount of the conjugate of claim 4, sufficient to provide protection against cholera, to a patient.

21. A method for immunizing a patient against cholera which comprises administering an amount of the conjugate of claim 9, sufficient to provide protection against cholera, to a patient.

FIGURE 1

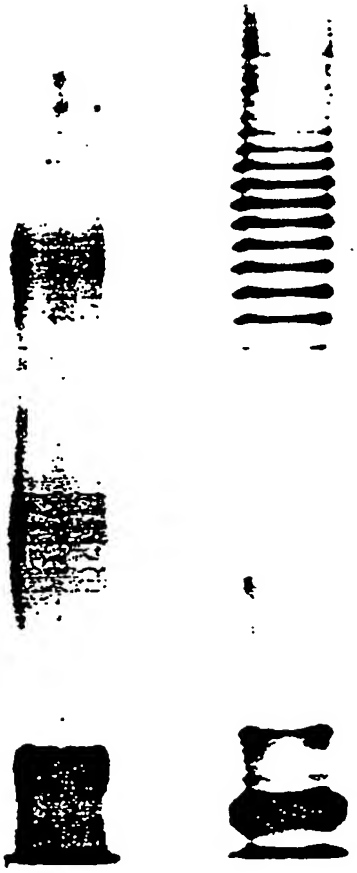


FIGURE 2

FIGURE 3

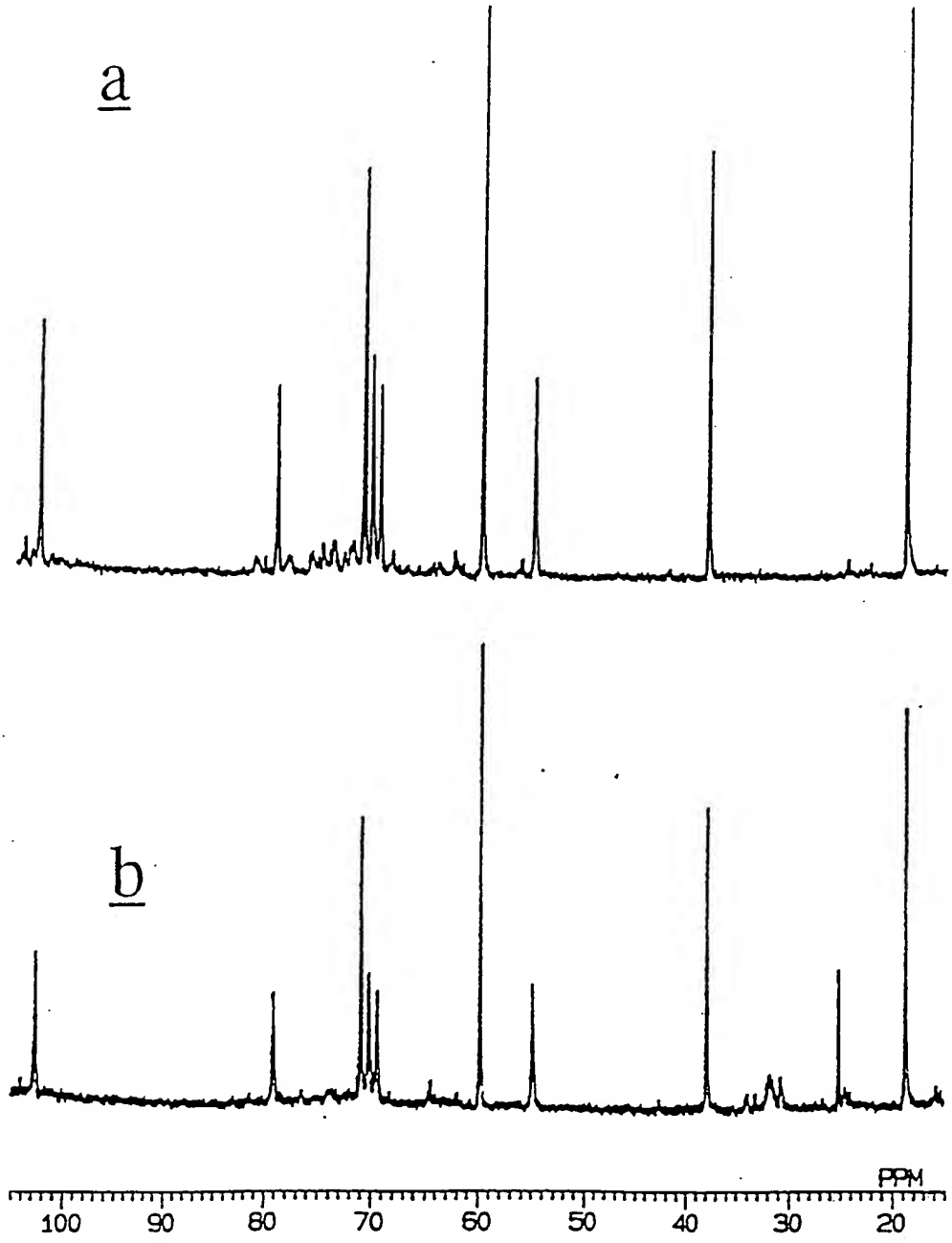
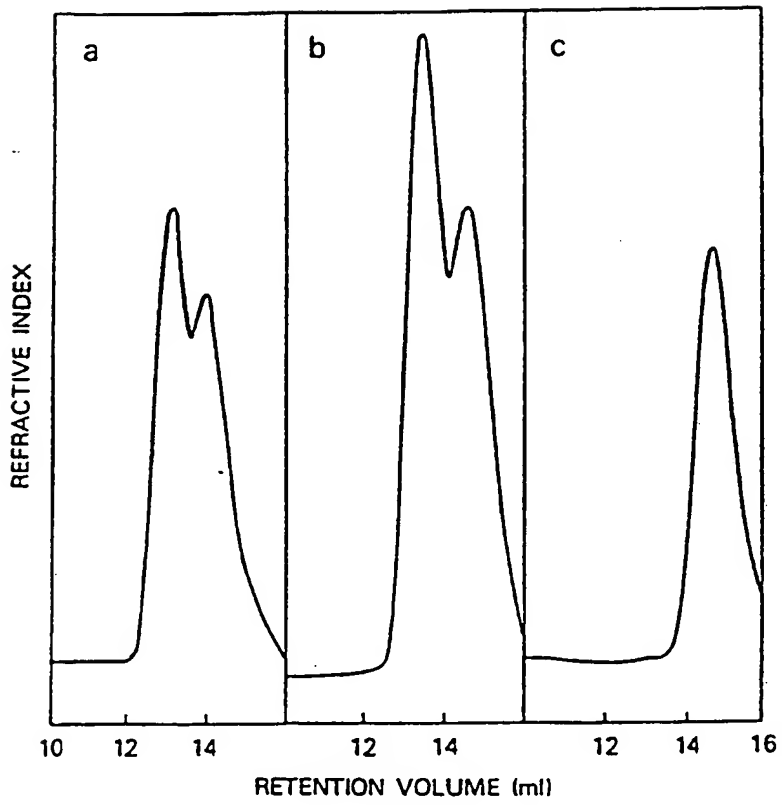


FIGURE 4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RETENTION VOLUME (ml)